

FEMALE SEXUAL BEHAVIOR AS THE MECHANISM RENDERING *Aedes Aegypti* REFRACTORY TO INSEMINATION¹

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The yellow fever mosquito, *Aedes aegypti* (L.), is one of the most thoroughly studied insects from the standpoint of both basic and applied science. Because of its importance as a vector of human diseases, a great deal of literature has accumulated concerning aspects of its systematics, distribution, bionomics, disease relationships and control. In spite of this intensive study, little was known until recently about its mating behavior. Recent research on sexual behavior of mosquitoes has indicated that many widely accepted concepts were incorrect. Moreover, several points of controversy remain.

One of the first serious investigations of sexual behavior in *A. aegypti* before and during coitus was undertaken by Roth (1948). In this paper he firmly established that flight sound of the female mosquito attracted males and initiated copulatory behavior. Later, Spielman (1964) and Jones and Wheeler (1965) described the act of copulation in detail and attempted to explain the mechanism by which semen is transferred by the male to the female.

Early students of the behavior of *A. aegypti* observed that females begin to copulate soon after emergence and continue to do so throughout life. Most assumed that the terms "mating," "copulation" and "insemination" were synonymous, thus concluding that most copulations result in insemination. MacGregor (1915) stated that copulation takes place as soon as females are ready to fly. Roth (1948) noted that most females are mated within 105 to 145 minutes after emergence, and recorded a single female that underwent 50 successful copulations by 11 different males during a one hour period. Finally, Gillett (1955) observed that, in captivity, a single female of *A. aegypti* may mate as many as 40 times before her first blood meal.

There has been considerable confusion and improper usage of terms affecting mosquito reproduction. The following is a partial list of terms related to behavior and reproduction as used herein:

Mating = That portion of sexual reproduction which begins with the search for a sexual partner and ends with successful insemination. Copulation or coitus represents only one portion of mating. Mating behavior includes swarming and other mate-finding mechanisms.

Copulation = The act of sexual intercourse. Coitus, coition, sexual union. In mosquitoes, copulation is a rather mechanical process. It requires the bodies of

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both partners to be in the correct position and their genitalia to be at the proper degree of connection so that ejaculation and insemination can take place.

Coupling = The events occurring when a sexually aroused male seizes a female and establishes genital contact. The male remains in contact and tries to achieve a position of coitus that will permit insemination. He may strive for the copulatory position but not achieve it. A male encountering a sexually refractory female may couple, but cannot copulate.

Insemination = The steps that occur between male ejaculation and deposition of sperm in the spermathecae. Sperm and seminal fluid are introduced into the bursa copulatrix of the female and, within a few minutes, sperm begin to migrate to the spermathecae where they are stored until the time of fertilization.

A major revision in our understanding of the sexual behavior of *A. aegypti* occurred when it was demonstrated that females of this species are monogamous (Craig, 1967; Spielman, Leahy and Skaff, 1967). Copulation by a mature virgin female results in insemination. Although the female appears to copulate with other males, she is refractory to a second insemination for life. Subsequent insemination is prevented through the action of a substance from the male accessory glands that is transferred to the female in the seminal fluid. This substance has been designated "matrone" (Fuchs, Craig and Hiss, 1968).

Virgin females can be rendered refractory to insemination by implants of accessory glands from adult males (Craig, 1967; Spielman *et al.*, 1967), by injection of an aqueous extract of glands (Craig, 1967), or by injection of material extracted from lyophilized whole bodies of males (Fuchs *et al.*, 1968). The partially purified substance, matrone, has been shown to be a protein (Fuchs, Craig and Despommier, 1969).

A second major revision of the life history of *A. aegypti* came with the description of a post-emergence refractory period (Gwadz and Craig, 1968; Lea, 1968). During this refractory period, which may last 36 to 60 hours, females may couple repeatedly, but they are not inseminated. Lea (1968) showed that the onset of sexual receptivity could be prevented by removal of the corpora allata and restored by gland implants. He concluded that sexual receptivity was influenced by juvenile hormone, a product of the corpora allata. Later, Gwadz, Lounibos and Craig (1971) demonstrated that the post-emergence refractory period could be reduced by more than 70% by the topical application of a synthetic analogue of juvenile hormone to pupae or teneral adults.

The mechanisms which permit a female of *A. aegypti* to couple without being inseminated have become a matter of controversy. Both young virgin females and previously inseminated females appear to copulate repeatedly but they are not inseminated. Craig (1967) felt that matrone might stimulate the inseminated female to hold her vaginal lips closed, thereby preventing the introduction of semen. Gwadz and Craig (1968) hypothesized that young virgin females are not inseminated because they withdraw their terminalia and prevent males from completing firm genital union.

On the other hand, Spielman *et al.* (1967, 1969) concluded that the explanations offered by Gwadz and Craig served only to describe some of the behavioral characteristics of females refractory to insemination. Spielman and co-workers

felt that these behavioral traits alone were not major barriers to insemination. It was their hypothesis that the failure of effective insemination was due in part to the physical condition of the bursa copulatrix of the female at the time of copulation. They attempted to show that young or previously inseminated females are unable to alter the consistency of the semen as it is introduced into the bursa. They felt that the seminal mass is expelled by the female or withdrawn by the male at the termination of coitus. They further contended that sperm and seminal fluid is transferred to females of all ages and sexual experience; however, a female is capable of retaining semen from only one copulation during her lifetime.

Observation on insemination capacity of males are relevant to this controversy. A male can inseminate 5 to 7 females but has little or no capability for sperm replenishment once he is depleted (Gwadz and Craig, 1970; Hauserman and Nijhout, in press). If, as Spielman contends, copulations with refractory females involve semen transfer and subsequent semen loss, this loss should be reflected as a reduction in the capacity of a male for further insemination. The experiment of Powell (Craig, 1967) shows that male capacity for insemination is not reduced after exposure to previously inseminated females. The present study was undertaken to amplify the preliminary results of Powell. Moreover, an attempt was made to analyze the nature of post-emergence and post-insemination refractory responses.

MATERIALS AND METHODS

All experiments were conducted with the Rock strain of *A. aegypti*. Rock is a relatively large, uniform, vigorous strain used in a number of laboratories and may be considered as characteristic of the type form of *A. aegypti aegypti*. This strain was originally obtained from D. W. Jenkins in 1959 and is the most commonly used strain at the Vector Biology Laboratory of the University of Notre Dame.

All rearing was conducted in accordance with the methods of Craig and VandeHey (1962). Larvae were reared in enamel pans in 2500 ml of tap water at a density of approximately 200 larvae per pan. Larvae were fed a suspension of liver powder (Nutritional Biochemicals Corporation, Cleveland, Ohio) at a concentration of 10 g powder per 1000 ml of tap water. The feeding schedule consisted of 10 ml of suspension on the day of egg hatching (day 0), 10 ml on day 1, and 20 ml each on days, 2, 3 and 4. Pupation began late on day 4 and was completed by day 5.

Care was taken to insure uniform growth and pupation, and pans were discarded if the larvae they contained failed to achieve greater than 95% pupation by the middle of day 5.

Sexes were separated by size and transferred to petri dishes (15 cm diameter) lined with moist paper towelling. These dishes were placed in gallon cardboard ice cream containers covered with bolting cloth. Adult emergence took place in these containers.

All rearing and experimentation was performed at $27 \pm 1^\circ \text{C}$ and $80 \pm 5\%$ relative humidity. Day length was 16 hours.

In experiments where the age of the female was of importance, emerging females were collected from the cages at hourly intervals. All females used in

these age-related studies were of known age ± 30 minutes. The ages of females used in all other studies were known with ± 12 hours. Newly emerged adults were transferred to gallon containers and maintained on apple slices as a source of food.

In experiments where previously inseminated females were required, 2–3 day old females were placed with a surplus of males for two days. This treatment resulted in approximately 95% insemination of females.

Determination of sexual receptivity

Females of *A. aegypti* do not go through any obvious precopulatory courtship behavior. Therefore, the criterion for sexual receptivity used herein was insemination of the female upon exposure to males. Copulation and insemination of receptive virgin females normally takes place within minutes of exposure; indeed, the entire act can be completed in less than 10 seconds. To determine if females are receptive or refractory to insemination, the following procedures were utilized. A group of 10 to 15 females was placed in a gallon cage containing 30 to 40 males of mixed ages from 2 to 10 days old. Exposure time was 24 hours. Females were then dissected in saline and their bursae and spermathecae examined for sperm with a compound microscope at $100\times$. Scoring for insemination was on the basis of presence or absence of sperm in the spermathecae. In a second method for determination of receptivity, individual females were exposed to 1 or 2 males in a modified lantern chimney 10×12 cm, instead of a gallon cage. The chimneys were rotated on their sides to induce mosquito flight until couplings occurred. Chimney rotation was stopped after a mosquito pair coupled and the duration of genital contact was recorded. The female was then removed and examined for insemination.

An apparatus was designed to allow observation of the behavioral responses of female mosquitoes to mating attempts by males. Individual females were lightly etherized and glued by the mesonotum to the head of a pin with a fast-drying plastic cement (Dekadhesse, Donald Tullock, Jr., Chadds Ford, Pennsylvania). After recovery from anaesthesia, the female was lowered into the viewing cage. Flight was stimulated by directing a stream of air over the female; copulation attempts by the free-flying males quickly ensued. Observation was simplified by viewing through a stereoscope mounted horizontally in front of the cage. The stereoscope had a magnification potential of from 10 to $70\times$ and was fitted with a photographic attachment.

Matrone preparation and assay

An extract of the active principle of male accessory glands, matrone, was prepared in accordance with the method described by Craig (1967) with minor modifications. Accessory glands were obtained from virgin males, 4 to 6 days old, by pulling the terminalia of these males while holding the thorax. One hundred tails in 1 ml of *A. aegypti* saline (Hayes, 1953) were sonicated for 10 seconds and subjected to -5°C centrifugation at 18,000 *g* for 20 minutes. The residue was discarded and the supernatant frozen until needed.

Assay procedures to determine activity consisted of: (1) intrathoracic injection of 20 virgin females with 1 μ l of solution, (2) 24 hour recovery period followed by a 24 hour exposure to a surplus of males, (3) examination of females for evidence of insemination. Active matrone renders more than 95% of the injected females refractory to insemination for life. Saline-injected control females show greater than 90% insemination after a 24 hour exposure to males.

Method for isotopic labelling

Third instar larvae (72 hours old) were transferred into a paper pint rearing cup containing 200 ml of water. An aqueous solution containing C^{14} was added to give a concentration of 0.05, or 0.10 microcuries per ml. The isotope used was either L-isoleucine- C^{14} or yeast protein (whole)- C^{14} , obtained respectively from New England Nuclear or the International Chemical and Nuclear Corp.

TABLE I
Radioactivity measurements of adult male mosquitoes from larvae reared in labelled solution from third instar to pupation

Treatment*	Conc. μ c/ml	Adult males			
		No. tested	Counts/minute		
			Minimum	Maximum	Mean
Isoleucine- C^{14}	0.05	10	14,610	62,277	27,681
Yeast protein- C^{14}	0.10	10	6,134	16,599	13,462

* 50 larvae, 72 hours old, placed in 200 ml water containing liver powder and isotope; larvae remained in this solution for three days, until pupation.

The regular larval feeding regime was continued; liver powder suspension was added to all containers. Each cup contained fifty larvae. The larvae remained in the labelled solution until pupation, generally a period of three days. Pupae were sexed according to size (males half as large as females) and transferred to emergence containers.

Assays of radioactivity were obtained using a Packard Tri-Carb liquid scintillation spectrometer (model 33651). The adult mosquitoes to be tested were crushed individually on a small piece of filter paper and were placed in 20 ml glass counting vials with foil-lined screw caps. The scintillation fluid was composed of 7 g of PPO (2,5-diphenyloxazole) and 50 mg of POPOP (p-Bis 2-(5-phenyloxazolyl)-benzene) per liter of toluene. Data are reported in counts per minute; the background count averaged 25 counts/minute.

Radioactivity of labelled males is indicated in Table I. Isoleucine- C^{14} at 0.05 μ c/ml gave males averaging 27,681 cpm, whereas yeast protein- C^{14} gave males with an average of 13,462 cpm. Perhaps the labelled amino acid was assimilated more rapidly, thus explaining the higher mean count from the less concentrated solution.

RESULTS

Sexual receptivity and sperm transfer

A single virgin male was exposed to 10 receptive virgin females in a gallon-sized cage for a 24 hour period, then placed with 10 additional females for a second 24 hours. After each exposure the females were dissected and examined for evidence of insemination. During the first 24 hour period, the 33 males tested inseminated an average of 5.70 females per male (Table II). However, during the second 24 hours these males were able to inseminate only 0.05 females per male, and none of these females received a full complement of seminal fluid. Obviously, these males had lost the capacity for further insemination. The females used for these latter exposures were refractory for one of three reasons: imma-

TABLE II

The effect of prior exposure to refractory females on male capacity for insemination. Each male was exposed to a different group of 10 females for each of 5 sequential 24 hour periods

No. ♂♂ tested	Types of refractory ♀♀ for exposure periods 1-3*	No. ♀♀ inseminated (of 10 available) per male in each period**				
		Refractory ♀♀			Mature virgin ♀♀	
		1	2	3	4***	5
33	None	----- no exposure -----			5.70 ± 1.24	0.05
29	Immature virgin	0	0	0	5.72 ± 1.36	0.17
28	Matrone-injected	0	0	0	5.61 ± 1.03	0.18
23	Previously inseminated	—	—	—	5.70 ± 1.29	0.13

* Follow exposure to refractory ♀♀ for periods 1-3, each ♂ was exposed to mature virgin ♀♀ for periods 4-5.

** Exposure periods, numbered 1-5, are 24 hours in length; exposure in gallon cage.

*** Mean ± S.D.

turity, previous insemination or matrone injection. Males initially exposed to young virgin females, previously inseminated females or matrone-injected females were subsequently capable of inseminating 5.72, 5.70 and 5.61 females per male respectively. There was no indication of semen loss as a result of previous exposure to refractory females.

Coupling in gallon cages over a 24 hour period could not be readily observed, so the experiments reported in Table II were repeated using a lantern chimney as the mating chamber (Table III). Under these conditions, individual copulations were observed and timed. A single virgin male was exposed to five refractory females (24 hours old or matrone-injected) individually and in sequence. Two couplings of at least 8 seconds were permitted with each female, and the females were immediately examined for insemination or the presence of sperm on the external genitalia. The same male was then exposed in sequence to 10 receptive virgin females and permitted one copulation with each female. Immediately after coupling these females were also examined for insemination. The control experiments consisted of the exposure of a single virgin male to 10 virgin females

in sequence, with one coupling per female. Thus, the entire process of testing one male required less than an hour.

As indicated in Table III, virgin males with no previous experience were able to inseminate an average of 6.5 females per male. Males after prior coupling with refractory females showed a similar, undiminished capacity. Males coupling first with young virgin or matrone-injected females inseminated an average of 6.5 and 6.4 females per male respectively. Again, coupling with refractory females had no effect on male insemination capacity. Moreover, there was no evidence of semen wastage after coupling. Of the 100 refractory females examined, not a single one had semen on the external genitalia.

The capacity for insemination of males exposed to females in gallon cages was slightly lower than the capacity of males exposed in lantern chimneys (about 5.7 *versus* 6.5). However, the mean capacities within experiments and between ex-

TABLE III

The effect of prior coupling with refractory females on male capacity for insemination. Each male was permitted two couplings with each of five refractory females, then allowed one coupling with each of 10 virgin females.

No. ♂♂ tested	Type of refractory female	No. ♀♀ inseminated per male*	
		Refractory, 5 ♀♀ provided in sequence	Mature virgin, 10 ♀♀ provided in sequence**
10	—	----- no exposure -----	6.5 ± 0.97
10	Immature virgin	0	6.5 ± 1.27
10	Matrone-injected	0	6.4 ± 1.35

* All couplings observed in lantern chimneys. Each male exposed in sequence to 5 refractory and 10 mature virgin ♀♀.

** Mean ± S.D.

periments were not significantly different. The results presented in Tables II and III are essentially identical.

To support the conclusion drawn from Tables II and III, an entirely different type of experiment was designed. Radioactive males were produced by rearing larvae in a solution containing C^{14} . Groups of 15 Rock females were exposed to 20 labelled males for a period of three days. Following exposure, females were anesthetized, crushed individually on a piece of filter paper, and placed in counting vials for radioactivity determination in the scintillation counter.

Level of radioactivity was determined for different kinds of females. Ten virgin females gave counts averaging 24.2 cpm; the average background was 25 cpm. Virgin females placed with labelled males gave much higher counts. In one experiment, 15 females gave individual counts ranging from 52.7 to 100.8, with a mean of 62.4 cpm. Females were examined for insemination prior to counting. In this experiment, all 15 females were inseminated. In another experiment, the following counts were obtained from 14 females: 20, 21, 46, 50, 57, 62, 65, 78, 92, 99, 102, 110, 114, 116. Dissection showed that the females with counts of 20 and 21 had not been inseminated. Thus, counts/minute of a given female readily indicated whether she had received semen from a male.

Table IV gives results of exposure of virgin and refractory females to labelled males. Males from two different strains, ROCK and DISTORTER, were used. The DISTORTER strain gives sex ratio distortion, with high frequency of males in the progeny (Hickey and Craig, 1966). Virgin females exposed to males of either strain showed a high count, indicating insemination. On the other hand, refractory females gave counts very close to the average background count. In fact, the refractory females resembled the control, virgin females with no exposure to males. These females were refractory because they had previously been inseminated, and hence rendered monogamous by matrone. Clearly, the labelled males did not pass any seminal material to the refractory females.

TABLE IV
Radioactivity transferred to unlabelled ROCK females exposed for a 3-day period to virgin labelled males from two different strains

Isotope labelling of ♂♂*		Mean counts/minute/ROCK ♀				
Material	Conc. in µc/ml	♀ virgin, no ♂ exposure (N = 10)	♀ exposed to ♂♂ (N = 15)			
			♀ virgin, mature		♀ refractory**	
			♂ ROCK	♂ DISTORTER	♂ ROCK	♂ DISTORTER
Isoleucine-C ¹⁴	0.05	24	62	83	28	26
Yeast Protein-C ¹⁴	0.10	25	53	52	28	28

* See Table I.

** ♀♀ previously exposed to ♂♂.

The results of the experiment with radioactive males (Table IV) fully confirm the earlier experiments (Tables II and III) which showed that prior exposure to refractory females did not diminish a male's capacity for insemination.

Observations of refractory and receptive behavior

Mating behavior of single females in suspended flight was observed with the aid of a stereoscopic microscope. Couplings resulting in insemination were markedly different from couplings by refractory females that did not result in insemination. Virgin females, 4 to 6 days old, were exposed to suspended flight to free-flying males; of 41 females tested, all but one were inseminated after a single coupling (Table V). Observations of these matings indicated that each coupling involved firm genital union and corresponded to the descriptions of copulation by Spielman (1964) and Jones and Wheeler (1965). Figure 1 pictures a male and female of *A. aegypti* engaged in copulation which resulted in insemination. The degree of genital contact of this successful copulation is illustrated in Figure 2.

The mean duration of genital contact for the 40 successful inseminations was 12.04 seconds (Table V). This average copulation time for receptive virgin females in suspended flight closely corresponds to the 12.1 second copulation time for receptive females in free flight in a lantern chimney. It would appear that suspension of the female had no discernable effect either on the duration of coupling

or on the probability of insemination. Moreover, insemination of a receptive virgin female was normally achieved by the first male able to grasp her legs and bring his claspers into position for copulation. Indeed, if the terminalia of the male succeeded in touching the terminalia of the female, complete copulation and insemination was assured.

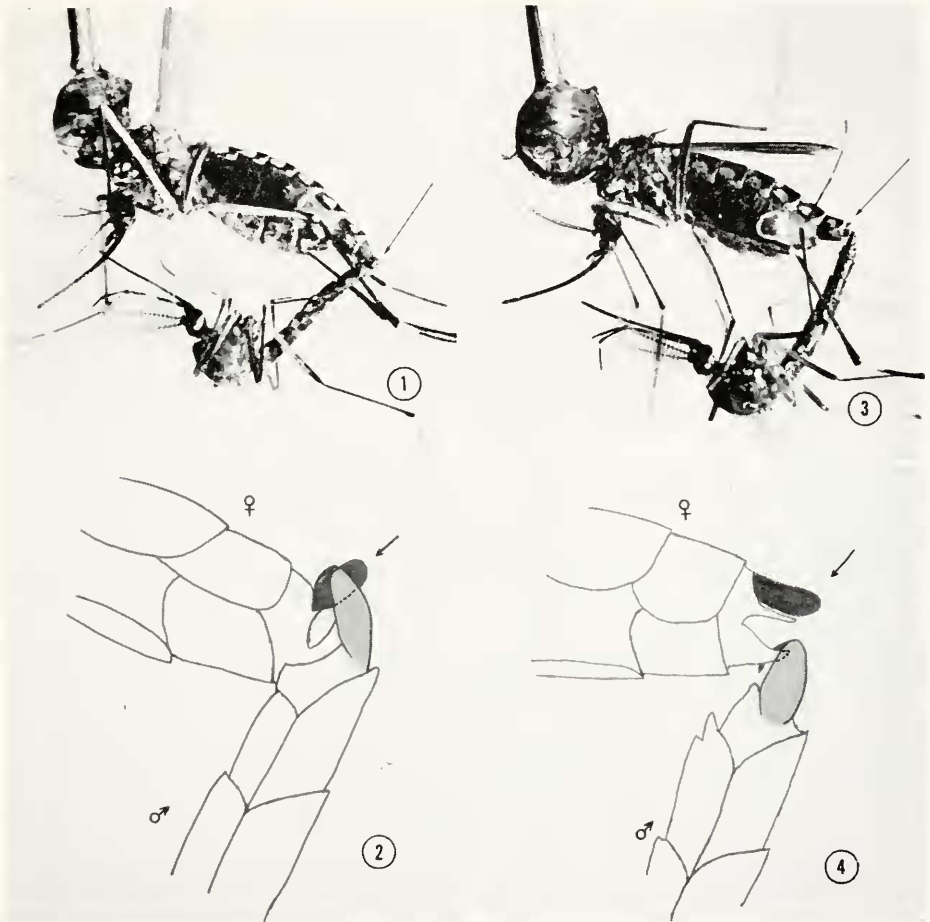


FIGURE 1. Copulation by a sexually mature female of *Aedes aegypti*. The arrow indicates the cerci of the female extending from beneath the claspers of the male. This copulation resulted in insemination of the female. The length of the male abdomen is approximately 3 mm.

FIGURE 2. Illustration of the degree of genital contact characteristic of copulation by a sexually receptive virgin female. The male claspers (shaded) fully engage the female cerci (dark).

FIGURE 3. Coupling without copulation by a sexually refractory female of *Aedes aegypti*. The arrow indicates the cerci of the female free and well above the claspers of the male. This coupling did not result in sperm transfer or insemination.

FIGURE 4. Illustration of the superficial genital contact typical of coupling by young refractory virgin females or previously inseminated females. The male claspers (shaded) do not touch the female cerci (dark). Copulation and insemination are impossible in this position.

Refractory females were exposed in suspended flight in the same manner as was employed with receptive females (Table V). Each refractory female was permitted a minimum of five couplings. None of 35 young tested actually copulated or were inseminated. Moreover, none of 25 matrone-injected females were inseminated. Obvious differences were noted in the behavior of refractory and receptive females. The duration of genital contact for couplings involving refractory females was extremely variable, with a range from 1 second to over 7 minutes; moreover, these copulations were never complete. Coupling by refractory females did not result in firm genital union (Figs. 3 and 4). The aedeagus of the male was so positioned that sperm transfer and insemination would be difficult if not impossible.

Visual determination of the state of receptivity of individual females is not difficult. An observer is able to differentiate between receptive and refractory

TABLE V

Receptivity to insemination of various types of females of Aedes aegypti in suspended flight. Individual females were suspended by the mesonotum and induced to fly in the presence of males; each coupling was observed and timed.

Female type	No. couplings allowed per ♀	No. ♀♀		Duration of genital contact in seconds
		Tested	Inseminated	
Mature virgin (4-6 days old)	1	41	40*	12.04 ± 3.30**
Matrone-injected	4-5	25	0	< 6***
Saline-injected	1	13	13	10.41 ± 2.51**
Young virgin (22-24 hrs.)	4-5	35	0	< 6***

* One unsuccessful coupling aborted at 6 seconds.

** Mean ± S.D.

*** Most couplings lasted less than 6 seconds; however, a few lasted over 60 seconds. Genital union was incomplete in all cases and no sperm was transferred.

females by noting the coupling activities of males. Males respond to sexually receptive females by ready contact, firm genital union and copulation. Males coupling with refractory females may be able to grasp the terminal sternite of the female but fail to copulate; they never achieve insertion of the aedeagus. These males may persist in their attempts to attain the proper coital position, but are never able to clasp the female cerci. The differences between the two types of coupling are so marked that females of unknown state can be determined as receptive or refractory on the basis of a single attempt if viewed with a stereoscope. However, to the unaided eye, both types of couplings appear to be normal copulations.

Speed of onset of refractory behavior

In order to determine the speed with which females change from receptive to refractory coupling responses, individual females were observed under conditions of suspended flight. Each female was permitted two couplings with free-flying males. Each coupling was observed and timed, and the interval between the first and the second coupling was noted. Eighteen mature virgin females were tested.

The first coupling of each female was characterized by a firmness of genital union typical of a successful copulation. None of the second couplings were successful. The behavior of females during the second coupling was comparable to the behavior of previously inseminated or matrone-injected females. Moreover, these females were refractory to repeated attempts by several males.

The interval between the first and second couplings varied from 7 to 261 seconds with a mean of 93 seconds. This interval was not a function of female receptivity but was related to the speed with which the second male was attracted to the suspended female.

On the basis of these observations it would appear that the switch from receptive to refractory behavior is an almost instantaneous result of insemination. The first coupling invariably resulted in a firm copulation. The second coupling was always superficial and typically refractory. Moreover, the switch is not the result of the act of copulation itself. Virgin females will copulate repeatedly with semen-depleted males. Obviously, no semen transfer can occur, yet these copulations always show firm genital union, however, these females remain sexually receptive and will immediately copulate with a fresh male.

DISCUSSION

Females of *A. aegypti* may couple repeatedly but they copulate only once, *i.e.*, semen is transferred only once. Consequently, they are monogamous. A female utilizes gametes from the insemination of a single male throughout her reproductive life. Only mature virgin females will copulate and can be inseminated. Both young virgin females and previously inseminated females are refractory to insemination; they may couple but do not copulate. Behavioral responses of the refractory female prevent firm genital union.

Spielman *et al.* (1967, 1969) felt that insemination of refractory females could not be prevented exclusively by behavioral factors. They felt that the condition of the bursa copulatrix of the female determined whether or not a female could be effectively inseminated. They froze females during and after forced-copulation and concluded that semen was transferred to females regardless of age or previous mating status. Only mature virgin females would retain the semen; refractory females ejected it from the bursa. Forcemated refractory females usually had internal masses of semen in the region of the genital atrium.

Unquestionably, refractory females can be inseminated by forced copulation (Craig, 1967; Akey and Jones, 1968; Gwadz and Craig, 1968). However the forced-copulation technique overrides the behavioral responses which prevent insemination under conditions of free flight. Therefore, data obtained using the forced-copulation technique are not relevant to normal insemination. We believe that our disagreement with the conclusions drawn by Spielman *et al.* (1967, 1969) are due in large part to their reliance on the forced-copulation technique.

Three lines of evidence from the present work bear out the contention that female behavior is adequate to prevent insemination of refractory females and that seminal expulsion is not involved. First, males exposed to refractory females showed no loss in insemination capacity. Males could inseminate 5-7 virgin females; repeated prior coupling with refractory females did not change this figure.

Secondly, refractory females exposed to males do not show external sperm masses. Of 100 refractory females examined, not a single one had semen on the external genitalia. Thirdly, refractory females placed with radioactive males showed no increase in radioactivity. If semen had been expelled from the bursa onto the external surface, the total count per female should have been raised.

From the standpoint of evolutionary parsimony, prevention of insemination of refractory females by seminal expulsion seems improbable. Considerable wastage of genetic material could occur. In our laboratory, Hausermann and Nijhout (in press) have shown that fecundity of male *A. aegypti* is strictly limited; a male cannot inseminate more than 5-7 females in a life-time. Yet, males of *A. aegypti* show hyperactive sexual activity (Roth, 1948), which would quickly lead to sperm depletion in the presence of refractory females. On the other hand, prevention of insemination by a behavioral mechanism of refractory females seems far more likely. If the female fails to give the proper cue indicating sexual receptivity, the male will not ejaculate and sperm will be conserved.

Spielman and his co-workers also argued that vacuolization of the bursal wall and spherule formation in the semen might be involved in the mechanism for semen retention. When seminal fluid comes in contact with a bursa *in vivo* or *in vitro*, physical changes do take place. However, there is no evidence to indicate that these events are in any way related to the retention of semen. Indeed, vacuolization and/or spherule formation are not always observed in females retaining semen (Spielman *et al.*, 1967, 1969).

Spielman concluded that the bursa of the mature virgin female (but not the immature or non-virgin female) has the capacity to "transform" seminal fluid and retain semen after copulation. However, Gwadz (1970) has shown that the copulatory behavior of female *A. aegypti* is regulated by the caudal sympathetic nervous system and is not related to the state of the bursa. When the terminal abdominal ganglion is surgically removed from a normally refractory female, she can be inseminated and will retain semen after copulation in free-flight. Although the chemical nature of the bursa of the young or previously inseminated refractory female is unchanged by the operation, the behavioral responses are altered. Normally refractory females that lack the terminal ganglion are not able to prevent copulation and insemination.

Sexual behavior of females of *A. aegypti* is similar in many respects to that displayed by females of the house fly, *Musca domestica*. Adams and Hintz (1969) have demonstrated a post-eclosion refractory period controlled by the corpora allata. Allatectomized female flies are mounted by males, but no insemination takes place because of female behavior. Moreover, house flies are monogamous; a component of the male seminal fluid renders females refractory to a second insemination for life (Riemann, Moen and Thorson, 1967). Sexually receptive females respond to courtship and mounting by extrusion of the ovipositor. This extrusion facilitates clasping and insemination by the male. Refractory females are mounted, but do not extrude the ovipositor; males are unable to clasp properly and cannot inseminate the female.

There are still serious gaps in our knowledge of mating behavior of female mosquitoes. The mode of action and precise target organs for the various hormones involved in regulating sexual behavior still need to be explored. More-

over, almost nothing is known about female pheromones, sex attractants and copulation initiators.

An even greater void exists in knowledge of the physiology of mating behavior of male mosquitoes. Because they are not disease vectors, males have received scant attention; their sex-related physiology has been virtually ignored.

Perhaps the greatest deficiency is in knowledge of mating behavior in the field. The where and when of mating, and the factors which bring the sexes together are questions of prime importance. The answers to these questions are especially relevant to those attempting programs of genetic control. These questions may prove to be among the most difficult to answer.

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SUMMARY

1. Certain females of *A. aegypti* are refractory to insemination, either because they are too young or because of previous insemination. Earlier reports indicated that refractory females prevent insemination by expulsion of semen from the bursa copulatrix. The present work indicates that this is not the case.

2. Three lines of evidence indicate that refractory females prevent insemination by failing to give the cues that lead to male ejaculation. First, males exposed to refractory females show no loss in their ability to inseminate. Secondly, refractory females exposed to males show no evidence of external sperm masses resulting from expulsion. Thirdly, refractory females placed with radioactive males show no increase in radioactivity, indicating no transfer of labelled semen. Since the forced-copulation technique overrides normal behavior of females, earlier data obtained using this technique are not relevant to normal insemination.

3. Copulation by a mature virgin female is characterized by firm genital union followed by insemination. A refractory female allows the male to couple but not to copulate. Coupling involves a superficial joining of the genitalia; no ejaculation or insemination occurs.

LITERATURE CITED

- ADAMS, T. S., AND A. M. HINTZ, 1969. Relationship of age, ovarian development, and the corpus allatum to mating in the housefly, *Musca domestica*. *J. Insect Physiol.*, **15**: 201-215.
- AKEY, D. H., AND J. C. JONES, 1968. Sexual responses of adult male *Aedes aegypti* using the forced-copulation method. *Biol. Bull.*, **135**: 445-453.
- CRAIG, G. B., JR., AND R. C. VANDEHEY, 1962. Genetic variability in *Aedes aegypti* (Diptera: Culicidae). I. Mutations affecting color pattern. *Ann. Entomol. Soc. Amer.*, **55**: 47-58.
- FUCHS, M. S., G. B. CRAIG, JR. AND D. D. DESPOMMIER, 1969. The protein nature of the substance inducing female monogamy in *Aedes aegypti*. *J. Insect. Physiol.*, **15**: 701-709.
- FUCHS, M. S., G. B. CRAIG, JR. AND E. A. HISS, 1968. The biochemical basis of female monogamy in mosquitoes. I. Extraction of the active principle from *Aedes aegypti*. *Life Sciences*, **7**: 835-839.
- GILLET, J. D., 1955. The inherited basis of variation in the hatching-response of *Aedes* eggs (Diptera: Culicidae). *Bull. Entomol. Res.*, **46**: 255-265.

- GWADZ, R. W., 1970. The neuro-hormonal regulation of sexual receptivity in female *Aedes aegypti* (Diptera: Culicidae). *Ph.D. thesis, University of Notre Dame*, 88 pp.
- GWADZ, R. W., AND G. B. CRAIG, JR., 1968. Sexual receptivity in female *Aedes aegypti*. *Mosq. News*, **28**: 586-592.
- GWADZ, R. W., AND G. B. CRAIG, JR., 1970. Female polygamy due to inadequate semen transfer in *Aedes aegypti*. *Mosq. News*, **30**: 355-360.
- GWADZ, R. W., L. P. LOUNIBOS AND G. B. CRAIG, 1971. Precocious sexual receptivity induced by a juvenile hormone analogue in females of the yellow fever mosquito, *Aedes aegypti*. *Gen. Comp. Endocrinol.*, in press.
- HAYES, R. O., 1953. Determination of a physiological saline solution for *Aedes aegypti* (L.). *J. Econ. Entomol.*, **46**: 624-627.
- HICKEY, W. A., AND G. B. CRAIG, JR., 1966. Genetic distortion of sex ratio in a mosquito, *Aedes aegypti*. *Genetics*, **53**: 1177-1196.
- JONES, J. C., AND R. E. WHEELER, 1965. An analytical study of coitus in *Aedes aegypti* (Linnaeus). *J. Morphol.*, **117**: 401-424.
- HAUSERMANN, W., AND H. F. NIJHOUT, 1970. Loss of male fecundity following sperm depletion in *Aedes aegypti*. *J. Med. Entomol.*, in press.
- LEA, A. O., 1968. Mating of virgin *Aedes aegypti* without insemination. *J. Insect Physiol.*, **14**: 305-308.
- MACGREGOR, M. E., 1915. Notes on the rearing of *Stegomyia fasciata* in London. *J. Trop. Med. Hyg.*, **18**: 193-196.
- RIEMANN, J. G., D. J. MOEN AND B. J. THORSON, 1967. Female monogamy and its control in houseflies. *J. Insect Physiol.*, **13**: 407-418.
- ROTH, L. M., 1948. A study of mosquito behavior. An experimental laboratory study of the sexual behavior of *Aedes aegypti* (Linnaeus). *Amer. Midland Natur.*, **40**: 265-352.
- SPIELMAN, A., 1964. The mechanics of copulation in *Aedes aegypti*. *Biol. Bull.*, **127**: 324-344.
- SPIELMAN, A., SR. M. G. LEAHY AND V. SKAFF, 1967. Seminal loss in repeatedly mated *Aedes aegypti*. *Biol. Bull.*, **132**: 404-412.
- SPIELMAN, A., SR. M. G. LEAHY AND V. SKAFF, 1969. Failure of effective insemination of young female *Aedes aegypti* mosquitoes. *J. Insect Physiol.*, **15**: 1471-1479.